RESEARCH ARTICLE

Genetic characterization of denitrifier communities with contrasting intrinsic functional traits

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Abstract

Microorganisms capable of denitrification are polyphyletic and exhibit distinct denitrification regulatory phenotypes (DRP), and thus, denitrification in soils could be controlled by community composition. In a companion study (Dörsch *et al.*, 2012) and preceding work, *ex situ* denitrification assays of three organic soils demonstrated profoundly different functional traits including N_2O/N_2 ratios. Here, we explored the composition of the underlying denitrifier communities by analyzing the abundance and structure of denitrification genes (*nirK*, *nirS*, and *nosZ*). The relative abundance of *nosZ* (vs. *nirK* + *nirS*) was similar for all communities, and hence, the low N_2O reductase activity in one of the soils was not because of the lack of organisms with this gene. Similarity in community composition between the soils was generally low for *nirK* and *nirS*, but not for *nosZ*. The community with the most robust denitrification (consistently low N_2O/N_2) had the highest diversity/richness of *nosZ* and *nirK*, but not of *nirS*. Contrary results found for a second soil agreed with impaired denitrification (low overall denitrification activity, high N_2O/N_2). In conclusion, differences in community composition and in the absolute abundance of denitrification genes clearly reflected the functional differences observed in laboratory studies and may shed light on differences in *in situ* N_2O emission of the soils.

Introduction

Denitrification is an alternative anaerobic respiration process reducing nitrogen oxides (nitrate and nitrite) stepwise to N_2 via the intermediates NO and N_2O (Zumft, 1997). This process completes the global nitrogen cycle and is of particular importance for the biogeochemical cycling of nitrogen in soils (Conrad, 1996; Philippot *et al.*, 2009). In soils, denitrification competes with plants for nitrate and is an important source for N_2O. Soil emissions contribute about 70% of the N_2O emitted to the atmosphere (Conrad, 1996), where it leads to the depletion of stratospheric ozone and contributes to the greenhouse effect (Crutzen, 1970; Dickinson & Cicerone, 1986). The microorganisms capable of denitrification are polyphyletic facultative organisms that shift from oxygen respiration to anaerobic respiration using nitrogen oxides as alternative terminal electron acceptors upon the transition from oxic to anoxic conditions. Low oxygen tension and the presence of nitrogen oxides are the prevailing exogenous signals inducing the synthesis of the denitrification system (van Spanning *et al.*, 2007). However, the regulatory networks of denitrification are variable, and strain-specific differences in the kinetics of denitrification gene expression in response to oxygen depletion and nitrogen oxides have been demonstrated (Ferguson, 1994; Ka *et al.*, 1997; Zumft, 1997; Cavigelli & Robertson, 2001; Bergaust *et al.*, 2008; Saleh-Lakha *et al.*, 2008; Miyahara *et al.*, 2010). As a result, denitrifiers vary in their ability to completely denitrify nitrate to N_2 and variable amounts of intermediates (nitrite, NO, and N_2O) may accumulate in response to environmental factors such as temperature, oxygen partial pressure, and pH (e.g. Saad & Conrad, 1993; Thomsen *et al.*, 1994; Bergaust *et al.*, 2008, 2010). Therefore, it was suggested that differences in the induction and activity of the denitrification system in individual strains could result in ecosystem-level differences in N_2O

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emission under certain conditions (Schimel & Gulledge, 1998). Moreover, the denitrification apparatus is considered as the modular assemblage of four partly independent respiratory systems (Zumft, 1997). This is reflected by the existence of partial denitrifiers with more or less severely truncated reduction pathways (e.g. Sorokin *et al.*, 2003; Simon *et al.*, 2004; Shapovalova *et al.*, 2008; Falk *et al.*, 2010). A significant fraction of cultured denitrifiers lacks a *nosZ* gene coding for N_2O reductase (Jones *et al.*, 2008). Hence, on the community level, a higher proportion of *nirK* + *nirS* compared to *nosZ* was related to differences in denitrification product ratios $[N_2O/(N_2O + N_2)]$, explaining site-specific differences in N_2O emissions in some soils (Philippot *et al.*, 2011). Thus, community composition can affect community and ecosystem functioning, and several studies reported that the function of denitrifier communities was correlated with differences in community structure and/or abundance (Cheneby *et al.*, 1998; Cavigelli & Robertson, 2000; Rich *et al.*, 2003; Bremer *et al.*, 2009; Hallin *et al.*, 2009; Kandeler *et al.*, 2009; Morales *et al.*, 2010). However, because others found no structure/abundance–function relationship (Boyle *et al.*, 2006; Dandie *et al.*, 2008; Miller *et al.*, 2008; Wertz *et al.*, 2009), a coupling of structure and function of denitrifier communities seems to be ecosystem-dependent (Rich & Myrold, 2004) and may be determined by not yet understood environmental factors (Wallenstein *et al.*, 2006).

In this study, we revisited three soils from distantly located regions in Finland (FIN), Germany (GER), and Sweden (SWE) that were studied previously within the GEFOS (Greenhouse gas Emission from Farmed Organic Soils) project (Klemedtsson *et al.*, 1999). The soils were under similar cultivation but differed in peat type, drainage history, and degradation state and had distinct characteristics, for example, with regard to mean annual temperature, carbon content, and pH (for more information see Dörsch *et al.*, 2012). Their estimated annual N_2O emissions were significantly different and ranged from 4.1 kg N_2O-N ha^{-1} in the Swedish soil (Klemedtsson *et al.*, 2009), 8.4 kg N_2O-N ha^{-1} in the Finnish soil (Maljanen *et al.*, 2003), and up to 14.6 kg N_2O-N ha^{-1} in the German soil (P. Dörsch, unpublished data). Marked physiological differences in denitrification response to anoxia (Holtan-Hartwig *et al.*, 2000) and low temperature (Holtan-Hartwig *et al.*, 2002; Dörsch & Bakken, 2004) had been observed for these soils in laboratory experiments, which prompted us to hypothesize that the denitrifier communities harbored by these soils were composed differently. The three soils differed markedly in native pH (GER = 5.4, SWE = 7.1, FIN = 6.0), which also could explain functional differences by direct pH control. We therefore explored direct pH effects on denitrifier functioning in a companion study exposing bacterial consortia extracted from the same three soils to two different pH levels (Dörsch *et al.*, 2012). Denitrification responses to pH were shown to be variable for the three communities, but consistent with the denitrification characteristics reported in previous studies, thus underlining that also the pH response of denitrification was community-specific. In the present study, we explored structure and abundance of the communities based on their nitrite reductase (*nirK*/*nirS*) and N_2O reductase (*nosZ*) genes as proxies for the ability of the communities to produce and reduce N_2O. The reduction of nitrite is considered as the key step of the process because NO is the first gaseous product that cannot be further assimilated by the organisms. This step that also distinguishes true denitrifiers from other nitrate reducing microorganisms is catalyzed by either a copper- or a cytochrome cd_1-containing nitrite reductase of which only one type occurs in a given strain. Both types of nitrite reductase genes, as well as the *nosZ* gene that encodes nitrous oxide reductase, have been frequently targeted by PCR-based cultivation-independent studies as functional marker genes to explore the composition and abundance of denitrifier communities in soils (Stres *et al.*, 2004; Throbäck *et al.*, 2004; Dell *et al.*, 2010; Morales *et al.*, 2010; Szukics *et al.*, 2010). Here, we PCR-amplified, cloned, and sequenced denitrification genes (*nirK*/*nirS* and *nosZ*) from the soils in Finland, Germany, and Sweden. Based on phylogenetic analyses, we evaluated differences in the diversity and composition of denitrification genotypes; in addition, denitrification genes were quantified by most probable number (MPN)-PCR. Finally, we discuss links between the genetic makeup of the communities and the physiological responses reported elsewhere (Holtan-Hartwig *et al.*, 2000, 2002; Dörsch & Bakken, 2004; Dörsch *et al.*, 2012).

Materials and methods

Soil samples

Soils were sampled from three Terric Histosols (FAO) used for agriculture in (1) Southern Germany (GER, a drained fen site at the 'Donaumoos', 48°40′N 11°04′E); (2) Finland (FIN, an experimental site at the Siikasalmi Research Station in East Finland; 62°55′N 29°30′E); and (3) Sweden (SWE, a farmland near Falköping, 58°20′N 13°30′E). The German field had been cultivated with Triticale and the Finnish and Swedish fields with barley (*Hordeum vulgare* L.) without fertilization during the last 3–4 years prior to sampling in 1998. Composite soil samples (12 cores) were taken from the plow layer (0–20 cm), sieved at 2 mm, and stored at 4 °C until

DNA extraction. Soil characteristics are given in Dörsch *et al.* (2012).

DNA extraction, PCR amplification, cloning, and sequencing

DNA was extracted from three replicate soil samples (0.5 g fresh weight soil) as described in Dörsch *et al.* (2012). The concentration of the DNA extracts was determined with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Paisley, UK) using the procedure described by the manufacturer. From the soil DNA extracts, gene fragments of *nirK*, *nirS*, and *nosZ* encoding copper- and cytochrome cd_1-containing nitrite reductase and nitrous oxide reductase, respectively, were PCR-amplified using primer pairs nirK1F-nirK5R, nirS1F-nirS6R, and Nos661F-Nos1773R (Braker *et al.*, 1998; Scala & Kerkhof, 1998). Although more recent versions of these primers exist, we found that, for instance, the sequence variability detected when using the nirS1F-nirS6R primer pair was comparable to that observed using the commonly applied *nirS* primers published by Throbäck *et al.* (2004) (unpublished data). Conditions for the amplification of *nirK* and *nirS* gene fragments were as published previously (Braker *et al.*, 2000), but for *nosZ*, a modified protocol was applied. It included ten initial 'touchdown' cycles during which the annealing temperature was decreased by 0.5 °C for each cycle with an initial annealing temperature of 59 °C. The remaining 20 cycles were performed at a constant annealing temperature of 56 °C. To minimize any heterogeneity between subsamples, three PCR products each were pooled and purified using the Wizard®SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany). Approximately 25 ng of the pooled amplicons (*nirK*, 514 bp; *nirS*, 890 bp; *nosZ*, 1131 bp) were taken for cloning with the pGEM®-T vector system (Promega).

Inserts from white colonies picked at random were screened for inserts of the proper length with vector-specific primers (M13 reverse/T7 promoter). Subsequently, both strands of the inserts were sequenced with vector-specific primers and the ABI BigDye terminator kit Version 1.1 (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and separated on an automated ABI 3100 DNA sequencer.

Phylogenetic analysis

Sequences (84 *nirK*, 82 *nirS*, and 115 *nosZ* clones) that were obtained from the Finnish, German, and Swedish soils and that represented the expected amplicons were aligned to sequences from public databases with the fast aligner feature in ARB (www.arb-home.de) and translated to amino acids. To establish NirK, NirS, and NosZ phylogenies, 1218, 1214, and 1113 deduced amino acid sequences from cultured and uncultured denitrifiers, respectively, were considered that were available from public databases at the time of the analysis. Phylogenetic analysis included 122, 146, and 294 deduced amino acid positions for NirK, NirS, and NosZ, respectively, with a minimum identity of 0% and a maximum identity of 100% but omitting insertions or deletions (indels) owing to ambiguous positional homology. Phylogenetic analyses were performed using ARB and the PHYLIP software package version 3.6a2.1 (Felsenstein, 1989). Trees were reconstructed using the distance-matrix-based neighbor-joining method with the Jones–Taylor–Thornton amino acid replacement model (ARB), and PROTPARS as well as maximum likelihood (PHYLIP). Clusters within the trees consisted of sequences that were consistently grouped together by any of the tree calculation algorithms (neighbor joining, parsimony, and maximum likelihood) applied. To avoid crowding of the trees, sequences from uncultured organisms except those generated within this study and closely related sequences from isolates of identical phylogenetic affiliation based on their 16S rRNA gene sequences were removed manually without changing the overall topology of the trees. Thus, bootstrap values cannot be shown.

Analysis of community diversity and similarity

The individual distance matrices for deduced amino acids of *nirK*, *nirS*, and *nosZ*, respectively, that were calculated in PHYLIP were taken as input to DOTUR [*D*istance-Based *OTU* and *R*ichness; (Schloss & Handelsman, 2005)] which was used to assign amino acid sequences to OTUs (operational taxonomic units). After pairwise comparison, OTUs were defined based on a distance of $< 10\%$ using the furthest neighbor algorithm with a precision of the distances of 0.01. The 10% distance level was arbitrarily chosen and could possibly be lowered, as indicated by Palmer *et al.* (2009) who claim that 80% minimum threshold similarity could be used for NosZ to indicate species-level OTUs. OTUs were taken by DOTUR to calculate rarefaction and the richness estimator Chao1 as well as the Shannon diversity index from each sample which allowed comparing the estimates across samples. ACE richness estimator and Simpson's diversity index were also calculated (data not shown), showing the same trends as Chao1 and Shannon diversity index, respectively, and thus confirming the results.

To elucidate whether observed structural differences between the libraries were attributable to differences in membership or to the abundance of individual members, the pairwise similarity of two communities was estimated using SONS [*S*hared *O*TUs and *S*imilarity; (Schloss &

Handelsman, 2006)]. As implicated in SONS, we determined (1) the actual fraction of sequences belonging to shared OTUs for each individual community; (2) the similarity of two communities based on the estimated fraction of sequences belonging to shared OTUs using the classical Jaccard similarity index (J_{abund}); (3) the actual fraction of shared OTUs in the individual communities; (4) the fraction of OTUs shared between two communities by the classical Jaccard similarity index (J_{class}); and (5) the community structure similarity index θ based on the estimated number of sequences belonging to OTUs shared between two communities as proposed by Yue *et al.* (2001). Venn diagrams were constructed for *nirK*-, *nirS*-, and *nosZ*-containing communities from each soil based on Chao1 richness estimates. Therefore, richness was estimated (1) for the individual community; (2) for the pairwise shared richness of two communities; and (3) by pooling two communities and estimating the fraction shared with the third community. Use of different data sets explains inconsistencies in OTU richness estimates, for example, as observed for NirS in the Swedish soil (Fig. 2b).

Quantification of denitrification genes

The copy numbers of the denitrification genes *nirK*, *nirS*, and *nosZ* in the soil DNA extracts were determined by an MPN-PCR procedure (Picard *et al.*, 1992). Briefly, soil DNA was diluted in triplicate 10-fold serial dilutions from which fragments of the denitrification genes *nirK*, *nirS*, and *nosZ* were amplified by PCR using the primers and procedures described above. Positive amplification of gene fragments was detected on agarose gels stained with ethidium bromide. Again, serial threefold dilutions were carried out in triplicate from each DNA solution that was 10-fold less dilute than the highest dilution yielding a PCR product, which were then subjected to PCR amplifications of *nirK*, *nirS*, and *nosZ* gene fragments. The MPN of denitrification genes was determined from the minimal number of targets that resulted in positive amplification with the help of Cochran tables (Cochran, 1950). The MPN of target in the initial purified DNA solution was calculated by multiplication with the dilution factor and estimated per gram dryweight of soil. Differences in copy numbers were statistically evaluated by ANOVA, and pairwise differences were unraveled by Tukey's HSD *post hoc* test.

Nucleotide sequence accession numbers

Sequences (*nirK*, *nirS*, and *nosZ*) have been deposited in the EMBL nucleotide sequence database and accession numbers AM419468 – 747.

Results

Phylogeny, richness, and diversity of *nirK*, *nirS*, and *nosZ*

nirK

The majority of the deduced amino acids from *nirK* clones (76 sequences) were grouped in two clusters (I and V) of the NirK tree (Fig. 1a), and both clusters contained clones from all three soils. The remaining sequences were placed in four distinct clusters (II, IV, VI, and VIII) and clones from different soils grouped in distinct clusters. Sequences from the German, Finnish, and Swedish soils belonged to five, 12, and 15 different OTUs, respectively. Of a total of 28 OTUs, 24 contained sequences from only one soil and only four OTUs were shared by sequences from more than one soil. Shared OTUs occurred only for the Finnish soil on the one hand and either the German or the Swedish soil on the other hand. Sequences from the German and the Swedish soils, however, were not affiliated with shared OTUs. Chao1 indicated the lowest NirK richness in the German soil (S_{Chao} = 6) and the highest richness in the Finnish soil (S_{Chao} = 48), and the difference was significant [nonoverlapping confidence intervals (Fig. 2a)]. Pairwise comparison of NirK richness between the Finnish and the Swedish soils and between the German and the Swedish soils showed overlapping 95% confidence intervals for the NirK richness and hence no significance. Community diversity was expressed as Shannon diversity index (Table 1), and rarefaction curves (Supporting Information, Fig. S1) were created that allow for the comparison of diversity using unequal samples sizes. Both approaches showed the highest NirK diversity in the Swedish soil ($H_{Shannon}$ = 2.36), an intermediate level of diversity for the Finnish soil ($H_{Shannon}$ = 2.09), and the lowest diversity in the German soil ($H_{Shannon}$ = 1.00) that was significantly lower than in the two other soils as indicated by nonoverlapping confidence intervals. Rarefaction analysis indicated that at least the libraries for the Finnish and the Swedish soils were not sufficiently sampled, and thus, the diversity was most likely underestimated (Fig. S1a).

nirS

A total of 82 sequences were clustered into seven different clusters in the NirS tree (Fig. 1b). An extremely low NirS richness (S_{Chao} = 2) and diversity ($H_{Shannon}$ = 0.17) were observed for the Swedish soil (Table 1, Fig. 2b). All but one sequence (24) from this soil belonged to a single OTU (OTU 1) within cluster I. Cluster I contained in addition a large fraction of sequences from the German

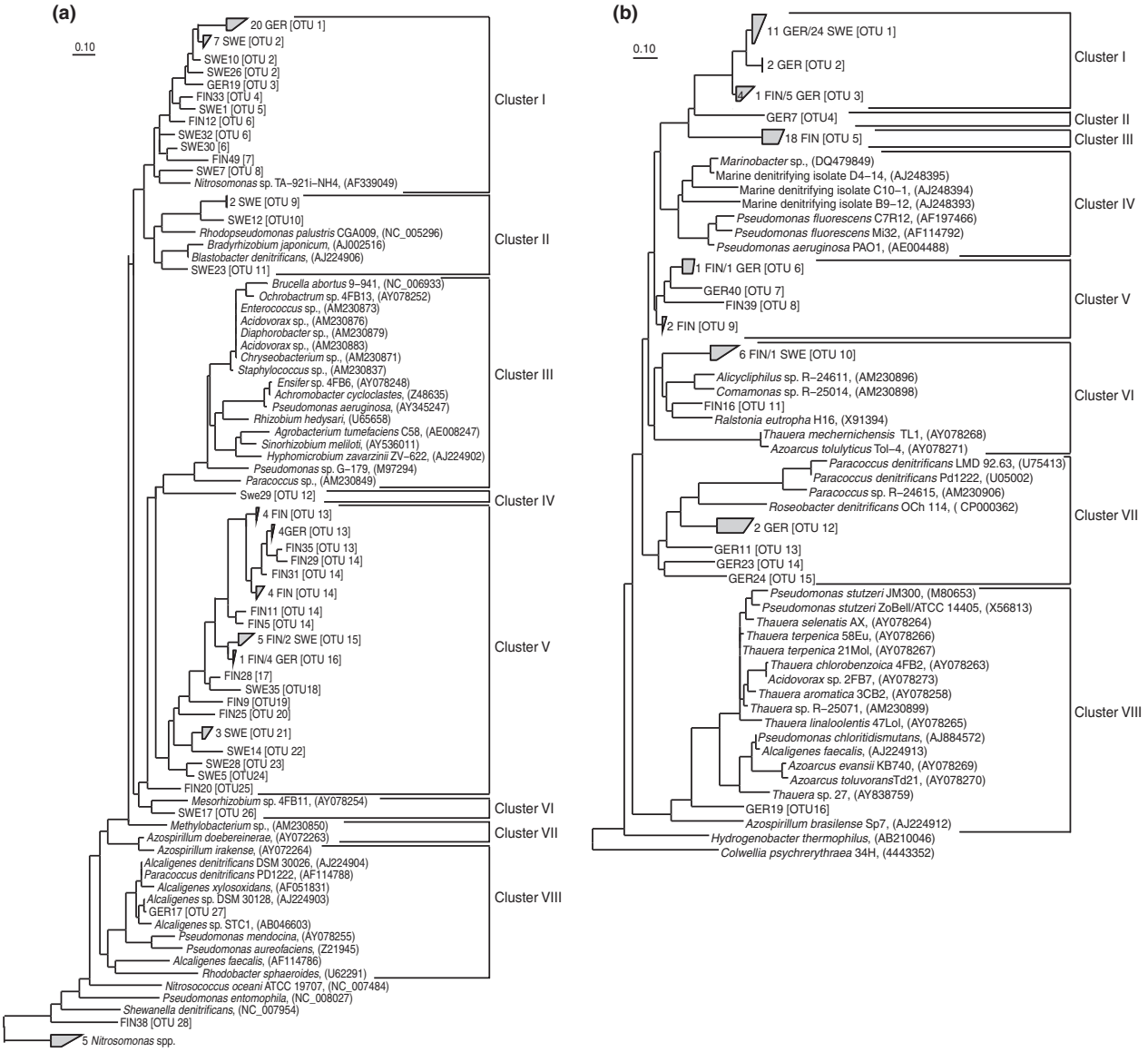

Fig. 1. Phylogenetic trees of deduced amino acids of PCR-amplified *nirK* (a), *nirS* (b), and *nosZ* (c) genes from soils from Finland (FIN), Germany (GER), and Sweden (SWE). The trees were reconstructed by the neighbor-joining algorithm with the Jones–Taylor–Thornton amino acid replacement model based on 122 (*nirK*), 146 (*nirS*), and 294 (*nosZ*) amino acid positions. Clusters were defined whenever sequences grouped consistently together when trees were calculated with different calculation methods. Operational taxonomic unit (OTU) designations are given in brackets, and numbers in front of clone designations indicate number of clones.

soil (11 sequences) also belonging to OTU 1, which was shared with the sequences from the Swedish soil. In the Swedish soil, only one additional singleton was found that was placed in a distinct cluster (cluster VI) together with sequences from the Finnish soil. Cluster III contained 18 sequences exclusively from the Finnish soil. In total, 16 different OTUs were detected for NirS sequences from all three soils. Four OTUs were shared among sequences from different soils, and 12 consisted of clones from only one soil but no OTU was shared between sequences from all three soils. The highest richness was estimated for the German soil (S_{Chao} = 18; Fig. 2b) and NirS diversity ($H_{Shannon}$ = 1.92) was also highest in this soil although rarefaction analysis showed that the *nirS* diversity in this soil was likely underestimated owing to nonsufficient sampling (Fig. S1b). Both estimators differed significantly from those of the Swedish soil but not from those of the Finnish soil.

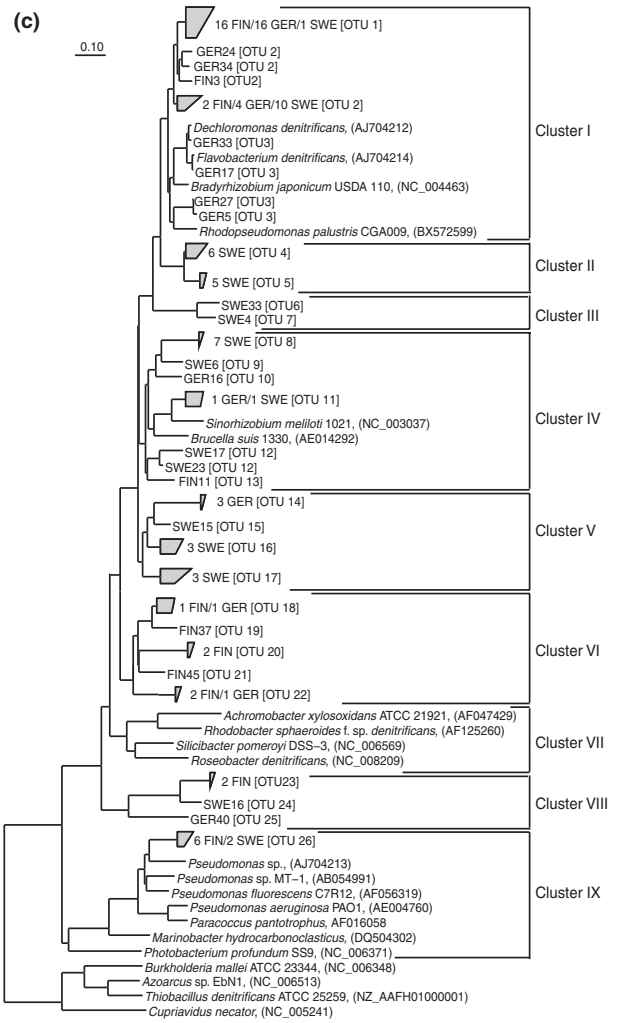

Fig. 1. Continued.

nosZ

The 114 NosZ sequences were grouped within eight clusters of the NosZ tree (Fig. 1c) and were assigned to 26 OTUs. Among these were 20 OTUs that were unique to one soil. Six OTUs were shared by sequences from more than one soil, and two OTUs even consisted of sequences from all three soils. Rarefaction analysis showed that sampling was fairly representative for community diversity because the curves started leveling off at least for the Finnish and German communities (Fig. S1c). Richness of the *nosZ*-containing denitrifier community was estimated to be highest in the Swedish soil (S_{Chao} = 22; Fig. 2c), intermediate in the German soil (S_{Chao} = 19), and lowest in the Finnish soil (S_{Chao} = 11), but the differences were nonsignificant. Diversity among NosZ OTUs was lowest in the German soil ($H_{Shannon}$ = 1.64; Table 1), significantly higher in the Swedish soil ($H_{Shannon}$ = 2.37), and intermediate in the Finnish soil ($H_{Shannon}$ = 1.81). However, the confidence intervals for the Shannon index indicated that NosZ diversity in the Finnish soil was not significantly different from that of the two other soils.

Similarity of denitrifier communities

nirK

The overall similarity of *nirK*-type denitrifier communities between the soils was very low. Communities from the German and Swedish soils were entirely distinct (θ = 0.00; Table 2) because no sequences occurred in shared OTUs (J_{abund} = 0), and hence, no OTUs were shared between the German and the Swedish soils (J_{class} = 0). The fraction of OTUs shared between the Finnish soil and either the German soil (J_{class} = 0.13) or the Swedish soil (J_{class} = 0.08) was also low. Estimating the OTU richness of each community and the richness shared between communities (Chao1) also showed that a major fraction of the OTUs in the Finnish soil but also in the Swedish soil was unique to these soils (Fig. 2a). Moreover, only a low fraction of sequences (22% and 14%, respectively) belonged to shared OTUs in each of these soils (Table 2). In contrast, 40% of the OTUs found in the German soil also contained sequences from the Finnish soil. However, the majority of *nirK* sequences from the German soil (20) belonged to OTU 1, which is unique to this soil, and therefore, the fraction of sequences belonging to OTUs shared with the Finnish soil was below 25%. Thus, the overall similarity between the *nirK*-type denitrifier communities in these soils was very low ($\theta \leq 0.05$) and so was the similarity between the communities of the Finnish and the Swedish soils ($\theta \leq 0.05$).

nirS

Estimates of OTU richness of individual soil communities and of shared richness of pairwise pooled communities indicated that the Swedish community was basically a subset of the Finnish and the German communities (Fig. 2b). The Swedish soil harbored only two OTUs of which OTU 1 was shared between communities of the German and the Swedish soils and the second (OTU 10) was shared between the communities of the Finnish and the Swedish soils (fraction of shared OTUs = 0.50; Table 2). A large fraction of sequences (96%) from the Swedish soil belonged to OTU 1 shared with the German soil. In addition, 41% of the sequences from the German soil belong to this OTU resulting in a comparably high J_{abund} value of 0.40. Hence, the most abundant members of both communities were shared in OTU 1, and there-

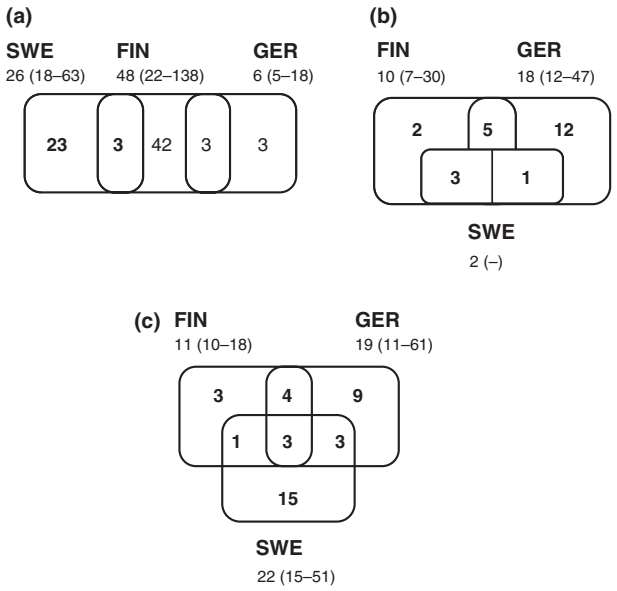

Fig. 2. Venn diagrams comparing estimated OTU memberships for deduced amino acids from *nirK* (a), *nirS* (b), and *nosZ* (c) gene libraries of the soils. Numbers below each soil indicate Chao1 richness estimate and 95% confidence interval for each community. Shared Chao1 richness was estimated for two communities and for two pooled communities with the third community.

Table 1. Comparison of *nirK*, *nirS*, and *nosZ* clone libraries of soils from Finland (FIN), Germany (GER), and Sweden (SWE) based on OTUs defined at the 10% distance level of deduced amino acids

Gene	Soil (No. of sequences)	No. of OTUs	No. of singletons	Shannon index
nirK	FIN (27)	12	9	2.09 (1.73–2.46)
	GER (29)	5	2	1.00 (0.62–1.37)
	SWE (28)	15	10	2.36 (1.97–2.75)
nirS	FIN (30)	7	4	1.26 (0.87–1.66)
	GER (27)	11	7	1.92 (1.50–2.33)
	SWE (25)	2	1	0.17 (0.00–0.68)
nosZ	FIN (35)	10	3	1.81 (1.46–2.16)
	GER (34)	9	5	1.64 (1.29–2.00)
	SWE (45)	15	7	2.37 (2.11–2.62)

fore, community structure similarity between *nirS*-containing communities in the German and the Swedish soils was estimated to be relatively large ($\theta = 0.52$). However, the community from the German soil showed a large fraction of OTUs of low membership that were unique to this soil, i.e. 12 OTUs of the 18 estimated OTUs in total (Fig. 2b), and only 13% ($J_{class} = 0.13$) and 8% ($J_{class} = 0.08$) of the OTUs were shared with the Finnish and the Swedish soils, respectively. Sequences within the single OTU that was found to be shared between the Finnish and the Swedish soils accounted for only 20% of the sequences from the Finnish soil. Abundance-based similarity between these two communities ($J_{abund} = 0.04$) as well as the fraction of OTUs shared between both soils ($J_{class} = 0.13$) was rather low, indicating that the community from the Finnish soil was numerically dominated by sequences that were not shared with the Swedish soil. It was even more dominated by sequences not shared with the German soil (93%), and the fraction of OTUs shared between both soils was low ($J_{class} = 0.13$). Thus, community structure similarity between the Finnish soil and the two other soils was very low ($\theta = 0.01$).

nosZ

In contrast to the *nirK*- and *nirS*-containing soil communities, community structure similarity was generally higher for the *nosZ*-containing soil communities (Table 2) with the highest similarity of $\theta = 0.76$ for the pairwise comparison between the Finnish and the German communities. A comparably high J_{abund} value of 0.56 and grouping of sequences into OTUs (Fig. 1c) indicated that the most abundant members of the communities from the Finnish and German soils were shared. Moreover, a comparably large fraction of 63% and 71% of the sequences of the individual communities belonged to shared OTUs. A large fraction of the sequences from the Finnish (71%) and German (68%) soils also belonged to OTUs shared between communities from these soils and the Swedish soil, respectively. Accordingly, richness estimates for OTUs of the individual communities and those shared between pairwise pooled communities showed a core community of three OTUs shared by all three soil communities (Fig. 2c). This core community, however, reflects only a minor fraction of genotypes found in the Swedish soil, while the majority of OTUs (80%) and a large part of the sequences (approximately 70%) were unique to this soil.

Abundance of denitrifier communities

The abundance of *nirK*, *nirS*, and *nosZ* gene copies in these soils was determined by MPN-PCR to allow using primers and PCR conditions identical to those used for community structure analysis. For *nirK*, copy numbers were 1–3.5 $\times$ 10^5 g^{-1} soil dryweight, with no significant difference between the soils (Fig. 3). For *nirS*, the copy numbers were 0.4, 1, and 5 ($\times 10^5$ g^{-1} soil dryweight) in the Finnish, German, and Swedish soils, respectively (all contrasts significant). The *nosZ* copy numbers were 1.9, 1.0, and 5.2 ($\times 10^5$ g^{-1} soil dryweight) in the Finnish, German, and Swedish soils, respectively. The only statistically significant difference, however, was that between the Swedish soil and the two others ($P = 0.035$).

Table 2. Community structure similarity of *nirK*-, *nirS*-, and *nosZ*-containing denitrifier communities in soils from Finland (FIN), Germany (GER), and Sweden (SWE) based on a level of 10% distance of deduced amino acids

	Community		Fraction of sequences belonging to shared OTUs in		Abundance-based similarity of	Fraction of OTUs shared in		Fraction of shared OTUs between	Community structure similarity
Gene	A	B	A	B	A and B (J_{abund})	A	B	A and B (J_{class})	θ
nirK	FIN	GER	0.22	0.24	0.14	0.17	0.40	0.13	0.05 (±0.03)
	FIN	SWE	0.22	0.14	0.11	0.17	0.13	0.08	0.05 (±0.04)
	GER	SWE	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (±0.00)
nirS	FIN	GER	0.07	0.22	0.07	0.29	0.18	0.13	0.01 (±0.01)
	FIN	SWE	0.20	0.04	0.04	0.14	0.50	0.13	0.01(±0.01)
	GER	SWE	0.41	0.96	0.40	0.09	0.50	0.08	0.52 (±0.14)
nosZ	FIN	GER	0.63	0.71	0.56	0.40	0.44	0.27	0.76 (±0.10)
	FIN	SWE	0.71	0.29	0.28	0.30	0.20	0.14	0.11 (±0.06)
	GER	SWE	0.68	0.27	0.28	0.33	0.20	0.14	0.14 (±0.07)

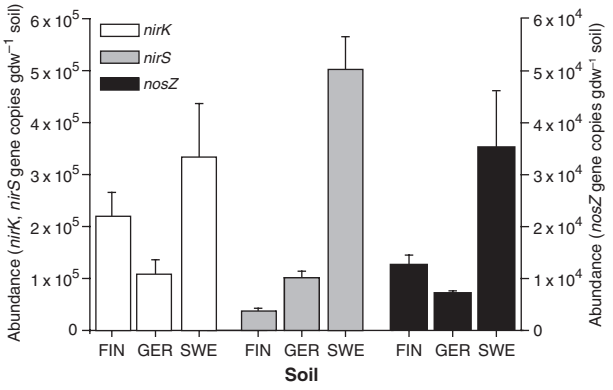

Fig. 3. Abundance of denitrification genes (*nirK*, *nirS*, and *nosZ*) in soils from Finland (FIN), Germany (GER), and Sweden (SWE) determined by MPN-PCR. Note different scales for *nirK*/*nirS* and *nosZ*. Values are means from three different DNA extracts ± SE.

Discussion

Functional characteristics in field, soil slurries, and extracted cells

The three soils emitted different amounts of N_2O as measured in field experiments, and a series of experiments with soil slurries suggested that this could be due to intrinsically different functionalities of the denitrifier communities involved. In laboratory experiments, the Swedish soil (low emissions of N_2O in field experiments) showed generally higher and not the least, earlier activity of N_2O reductase (N_2OR) than the other soils, resulting in lower transient accumulation of N_2O (Holtan-Hartwig *et al.*, 2000). The German soil generally had the lowest N_2OR activity under all conditions tested (Holtan-Hartwig *et al.*, 2000, 2002; Dörsch & Bakken, 2004). The Finnish soil was intermediate in both respects. All soils, however, showed a potential to increase N_2OR activity upon prolonged exposure to anoxia (48-hr preincubation). N_2O reduction rates increased to high values even in the German soil, although the relative activity of N_2OR (vs. that of NIR + NOR) was consistently higher in the Swedish than in the German soil (Table 2 in Holtan-Hartwig *et al.*, 2000). Further investigations effectively ruled out that the contrasts had anything to do with differences in the substrate affinity or temperature response (apparent activation energy) of the expressed N_2O reductase in the three soils (Holtan-Hartwig *et al.*, 2002). This indicated that all soils were able to express N_2O reductase with similar kinetic characteristics, but that their capacities to do so efficiently and at an early stage of an anoxic spell were very different. This implies that the essential difference between the communities lies in their kinetics of regulation.

In a companion study in which extracted cells from each soil were exposed to two different pH levels (5.4 and 7.1; Dörsch *et al.*, 2012), we effectively ruled out that differences in the denitrification kinetics between the soils were attributable to direct pH control. The experiments with extracted cells largely corroborated the contrasts observed in soil slurries and unraveled even more profound contrasts between the functional characteristics of the communities (response to pH both regarding N_2OR activity and transient accumulation of nitrite; see Dörsch *et al.*, 2012, for details). Taken together, these experiments give evidence that the three soils harbor communities with profoundly different abilities to conduct efficient and balanced expression of denitrification. In terms of denitrification efficiency, the German community scored lowest with clear signs of dysfunctionality (low activity, accumulation of large amounts of nitrite), whereas the Swedish

community was overly robust and essentially unaffected by environmental factors known to induce unbalanced denitrification (low temperature and low pH). The Finnish denitrifier community was intermediate in most respects.

Why did the three soils harbor such widely different functional denitrifier communities?

The soils differed in cultivation history and pedoclimatic origin. The German soil has most likely been acidic when drainage started in 1775, the Swedish soil has never been acidic since cultivated (naturally limed), whereas the pH history of the Finnish soil is more uncertain (shallow peat layer on a clay sediment, with some mixing through tillage). The long-term effect of the contrasting soil properties may possibly be of importance in selecting for certain phenotypes in the three soils. Carbon content, temperature, and pH in addition to oxygen and nitrate availability are known regulators of denitrification (Nömmik, 1956; Tiedje, 1988), and for this reason, they may also affect the denitrifier community composition in a longer term. Soil pH is known to generally affect microbial community diversity and richness in the long term (Fierer & Jackson, 2006) and denitrifier abundance and community composition in particular (Enwall *et al.*, 2005; Hallin *et al.*, 2009; Cuhel *et al.*, 2010). However, differences in cultivation (e.g. tilling, plant cover, and fertilizer application) as well as temperature also caused denitrifier community shifts in other studies (e.g. Avrahami *et al.*, 2002; Bremer *et al.*, 2007, 2009; Braker *et al.*, 2010; Cuhel *et al.*, 2010; Szukics *et al.*, 2010; Attard *et al.*, 2011). Hence, the distinct edaphic factors of the soils strongly suggested intrinsic differences in denitrifier community composition.

We evaluated differences in the genetic makeup of the communities by comparing deduced amino acid sequence libraries for denitrification genes encoding nitrite and nitrous oxide reductase (*nirK*, *nirS*, and *nosZ*) via ∫-Libshuff (Schloss *et al.*, 2004). This analysis indicated statistically significant pairwise differences in the structure of the *nirK*-, *nirS*-, and *nosZ*-containing communities from these soils ($P < 0.0017$; data not shown). Community differences were further confirmed by UniFrac analysis ($P < 0.03$; data not shown) (Lozupone & Knight, 2005). However, neither of these approaches allows an analysis of community composition based on distinct levels of amino acid distance, and hence, no detailed characterization of structural differences can be deduced. Therefore, differences between denitrifier communities were extracted by DOTUR (Schloss & Handelsman, 2005) and SONS (Schloss & Handelsman, 2006). The results supported our hypothesis of profound differences in community composition although a complex picture of denitrifier community similarity emerged depending on which functional denitrification gene was evaluated. With respect to the diversity of *nirK*- and *nosZ*-type denitrifier communities, similar trends emerged with the highest levels in the Swedish soil, intermediate levels in the Finnish soil, but lowest levels in the German soil. Surprisingly, the *nirS*-type denitrifier communities in the Swedish soil did not follow this trend and showed an extremely low richness compared to the two other soils. The absolute abundance of denitrification genes in the soils was variable, but ratios between the abundance of *nosZ* and that of *nirK* + *nirS* for the three soils were very similar (0.032, 0.050, and 0.040 for the German, Finnish, and Swedish soils, respectively). The low ratios could be due to a bias by the method used (MPN-PCR-based estimates are sensitive to minor differences in efficiency of the PCR for each gene), but this bias (lower efficiency for *nosZ* than for the other genes) would probably be the same for all communities; hence, the near equal *nosZ*/(*nirK* + *nirS*) of the three soils suggest that they are fairly equal with respect to the relative number of *nosZ* genes.

Can we identify links between community composition and functional traits?

It has been suggested that a low relative abundance of *nosZ* in a denitrifier community is attributable to a higher abundance of denitrifiers with truncated denitrification pathways (lacking *nosZ*) and that this could result in higher $N_2O/N_2O + N_2$ ratios and N_2O emissions (Cuhel *et al.*, 2010; Philippot *et al.*, 2011). Our results suggest that this may be an oversimplification. The denitrifier communities studied here showed a range of contrasting responses to temperature and pH which cannot be ascribed solely to a high frequency of organisms without *nosZ* (Holtan-Hartwig *et al.*, 2002; Dörsch & Bakken, 2004; Dörsch *et al.*, 2012). It appears more likely that the contrasting performance of the communities is attributable to different regulation of denitrification by the dominating species within the three communities.

As hypothesized, we found profoundly different composition of the three denitrifying communities, suggesting that there is a causal relationship between composition and performance. However, it seems unlikely that this causal relationship results in consistent patterns of genotype/phylotype–function relationships in denitrifying communities. Sequences grouped within single OTUs may reflect some physiological similarity, but this appears less than likely for their regulation of denitrification. Even phylogenetically closely related organisms frequently show distinct denitrification phenotypes, supposedly due to the large number of genes involved in the regulation of denitrification (Fesefeldt *et al.*, 1998; Hashimoto *et al.*, 2009; Jones *et al.*, 2011). For instance, NirK sequences within

cluster I (Fig. 1a) most likely originate from slow-growing diazotrophic *Bradyrhizobia*, but these organisms differ in their ability to completely reduce nitrate to N_2 (Hashimoto *et al.*, 2009; Falk *et al.*, 2010). Moreover, there seems to be little congruence between the phylogeny of functional denitrification genes and the phylogenetic affiliation of the respective organisms owing to a variety of evolutionary mechanisms (Heylen *et al.*, 2006; Dandie *et al.*, 2007; Jones *et al.*, 2008).

Thus, although we cannot ascribe the functional traits of the three soil communities to the dominance of any particular group, the results can be considered a valuable hypothesis-generating data set and a point of reference for future studies of genotype/phenotype studies of single strains. It thus appears worth the effort to summarize some possible causal relationships:

(1) The marked functional differences between the Swedish and German denitrifier communities appeared to be linked to *nirK* and *nosZ* diversity as well as to the uniqueness of *nirK* and *nosZ* genotypes in the Swedish soil. The overall better performance of the Swedish soil agrees well with the general belief that species-rich microbial communities are more robust (Wohl *et al.*, 2004; Bell *et al.*, 2005). Diversity *per se*, however, is presumably not decisive for the function in question (here: denitrification), underlining the importance of unique members within the community (Salles *et al.*, 2009). N_2O reduction rates in the Swedish soil were almost invariably higher than nitrate and nitrite reduction rates in all experiments. This could be due to a dominance of strains which express higher N_2OR activity than that of the other enzymes, but this property may be unrelated to the phylogeny of the organisms.

(2) In the case of the acid-degraded German soil, a large fraction of the denitrification genotypes may represent organisms, regulatory networks, or at least enzymes that are adapted to the prevalent conditions in this soil. Compared to the other two soils, *nirS*-type denitrifiers showed the highest richness, diversity, and uniqueness in membership. The initial denitrification rate in the community from the German soil was higher when incubated at the native pH (5.4) than at pH 7.1 (Dörsch *et al.*, 2012), suggesting that there is a selection of acid tolerant strains as it was suggested by Palmer *et al.* (2010) and Cuhel & Simek (2011).

(3) The functional characteristics of the communities changed during the rather lengthy anoxic incubation (100 h), particularly so for the German community (retardation of the denitrification activity by high pH only lasted through the first 40 h; Dörsch *et al.*, 2012), and in all incubations, the kinetics of denitrification suggested substantial growth. Thus, the observed denitrification toward the end of the incubation was possibly dominated by organisms that were numerically low in the original inoculum. To explore whether a large part of the denitrification capacity of the German community at low pH indeed depended on denitrifiers specifically adapted to acidic pH, additional experiments studying the expression of denitrification genes under pH stress are on their way.

(4) Among the three soils, denitrifiers of the Finnish soil showed intermediate levels with respect to community composition (abundance of *nirK* and *nosZ*, diversity of all three genes) and nominally reflected a regular denitrification phenotype with balanced temperature response and an N_2OR response to pH equivalent to that found in other soils [low denitrification but high N_2O/N_2 product ratio at low pH (Liu *et al.*, 2010)].

Conclusions

Denitrifier communities from the three sites had widely different composition, with apparent implications for their functional characteristics and the propensity of the soils to emit N_2O. It would be premature to try to identify a relationship between structural components (specific gene sequences) and functional traits. In fact, it seems far from obvious that there exists a relationship between diversity and function, although the data for *nirK* and *nosZ* (but not for *nirS*) suggest a positive relationship between robustness and diversity. We found recurrent functional responses across a number of physiological experiments, which points toward specific denitrification regulatory phenotypes anchored in the taxonomic composition of denitrifier communities. Further progress in understanding the relationship between functional traits and species composition of communities may be achieved by exploring the relationship between genotype and regulatory phenotypes of a large range of ecologically relevant organisms, as suggested by Bergaust *et al.* (2011).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction analysis for deduced amino acid sequences of denitrification genes *nirK* (a), *nirS* (b), and *nosZ* (c) cloned from soils from soils from (FIN), Germany (GER), and Sweden (SWE) at a level of 10% genetic distance.

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